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Non-toxic synthetic adjuvants are under prime consideration for use in increasing the immune response of human beings. Three of microbial origin are the muramyl di-peptides (N-acetyl-muramyl-L-alanyl-D-isoglutamine and analogs, termed MDP), polyadenylic acid-polyuridylic acid complexes termed poly A·poly U) and the recently isolated monophosphoryl lipid A (termed MPL). While each of these has been demonstrated in animals to be active in increasing the immune response when given with the antigen, each also has been found to suppress this response when given one to several days before antigen. The enhancing actions of MDP and poly A·poly U have been well characterized. However, characterization of the suppressive phenomenon has been minimal, but is important to gain a responsible understanding of how these adjuvants regulate the immune response non-specifically. Accordingly, the experiments proposed during the tenure of this contract were undertaken to further our knowledge of how each of these adjuvants activate the suppressive arm of the immune response. The data are submitted in the form of four preprints and one reprint and

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permit the following main conclusions:

POLY A · POLY U

1. Poly A·poly inhibited antibody forming cells non-specifically when given 1-4 days before antigen, whereas poly I·poly C inhibited when given 1-6 days before antigen.

2. This suppression could be expressed in in vitro experiments by addition of surprisingly either T cell rich, B cell rich or adherent cell populations to their syngeneic normal cell counterparts, suggesting an unidentified cell may be contaminating each.

3. To determine whether an NK cell was contaminating the above 3 populations and was responsible for suppression, NK activity was removed with anti-asialo GM1 antibody without affecting the magnitude of the suppression.

4. Suppressive activity for both humoral and cell mediated immunity (MLR) was found and characterized in the serum of mice injected with poly A·poly U after 90 minutes.

5. Poly A·poly U increased non-specific resistance to Streptococcus pneumoniae and Pseudomonas aeruginosa when given 1-2 days before challenge with these microorganisms, despite the presence of antibody suppressing activity in the spleen at this time.

MURAMYL DI-PEPTIDES

1. A single injection of MDP either ip or iv, 1-2 days before antigen inhibited antibody forming cells by approximately 50%. This suppression lasted from 4-14 days with much individual variation.

2. Using derivatives of MDP it was shown that the muramyl grouping was not necessary for suppressive activity. The addition of an n-butyl ester grouping to the terminal carboxyl of the glutamine moiety of MDP did not increase the capacity to induce suppression.

3. Suppression could be transferred to syngeneic recipient mice with both adherent and non-adherent spleen cells. T cells were found to be the effector cell in the latter population.

4. Unlike poly A·poly U, MDP did not induce suppressive activity in the serum 90 minutes after injection.

5. Interleukin I activity was depressed 24 and 48 hr after MDP injection, while Il-2 activity became depressed later at 72 hr.

6. It was hypothesized that MDP initiates suppression in the macrophage population in the form of decreased Il-1 production, which in turn depressed Il-2 levels. The net result was a decrease in numbers of antibody forming cells.

MONOPHOSPHORYL LIPID A

1. A non-toxic monophosphoryl lipid A (MPL, Ribi) isolated from endotoxins of Gram negative bacteria was shown to exert an adjuvant action on both the helper and suppressor branches of the immune response. Thus, toxicity is not a requirement for the adjuvant action of bacterial endotoxins.

2. MPL restored antibody production in aging mice and in the endotoxin low responding mouse strains C3H/HeJ and C57B110/ScN. In addition, MPL induced suppression in the C3H/HeJ strain.

GENERAL

1. Poly A·poly U, MDP and LPS increased phagocytosis in macrophages from young mice, but appeared to suppress this activity in aging virgin mice.

2. Aging breeder mice on the other hand were activated to increased phagocytosis similar to young mice, suggesting hormonal factors may control certain reactivities to adjuvants.

The Adjuvant Properties of a Non-Toxic Monophosphoryl Lipid A

in Hyporesponsive and Aging Mice

by

Mark Tomai, Arthur G. Johnson, John Cantrell,

Terry Ulrich and Edgar Ribi

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Abstract

>The immunomodulatory action of a non-toxic monophosphoryl lipid A (MPL) and a toxic diphosphoryl lipid A (DPL) fraction derived from endotoxins of the heptoseless mutants of bacteria were compared. Both derivatives retained the ability characteristic of lipopolysaccharides, to enhance antibody formation in young adult mice when injected along with antigen and suppress antibody production when given a day before antigen. ¯In aging mice, a model of immunodeficiency, a marked restoration of antibody formation was observed when antigen was injected together with either MPL or DPL. Levels of antibody in these aging mice were comparable to those observed in young adult mice. Moreover, both MPL and DPL enhanced antibody production significantly in the endotoxin low-responder mouse strains, C3H/HeJ and C57B1/10 ScN, whereas, ⁓phenol-water extracted endotoxin from an R-7 mutant was ineffective. MPL and DPL also acted as suppressive agents when administered prior to antigen in the .C3H/HeJ strain. Thus, the results from these studies show that (a) the toxic properties of lipid A can be removed without eliminating immunomodulating activity, and (b) such lipidic compounds can overcome the immunologic lesions of immunodeficient and hyporesponsive animals. Originator Supplied Keywords include;

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Introduction

Although Gram negative bacterial endotoxins and their active component, lipid A, are toxic, they inherently possess immunomodulating characteristics (1), including potent adjuvant activity when injected with antigen (2-4), and suppressive activity when injected before antigen (5-7). They also synergistically enhance anti-tumor activity of the mycobacterial cell wall skeleton (CWS) and possess mitogenic activity for murine spleen cell cultures (8). Although, previous attempts have been made to detoxify the endotoxins

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and still retain their beneficial properties (9-12), routine clinical use of lipid A in immunotherapy has yet to be realized.

Recently, two chemically well defined fractions of low molecular weight have been prepared from the parent endotoxins. A diphosphoryl lipid A (DPL, 1790 Da) has been shown to be toxic by multiple parameters (13-16). In contrast, a monophosphoryl lipid A (MPL, 1717 Da), which lacks the reducing sugar phosphate occurring at the 1 pc-'cion in DPL, was found to be non-toxic while still retaining certain beneficial properties, e.g. acting as a potent spleen cell mitogen and enhancing CWS anti-tumor activity (8). Comparison of these two compounds should facilitate structure-function relationships. To evaluate whether these different, but chemically well defined lipid A's were also able to enhance and suppress antibody formation several experimental models were employed. The experiments described herein tested their immunomodulating activities in (a) young, adult mice; (b) the C3H/HeJ and C57Bl 10 ScN strains of mice which possess chromosomal mutations that render them hyporesponsive to the activities of lipopolypolysaccharide (17); and (c) aging mice, a model of immunodeficiency, in which only minimal amounts of antibody are produced when compared with young adult mice (18).

The data presented show that both the mono and diphosphoryl lipid A's are good adjuvants, able to enhance and suppress antibody production in aging as well as young adult mice. In addition, the mono and diphosphoryl derivatives enhanced antibody formation in the endotoxin low responding C3H/HeJ and C57Bl/10 ScN mice, whereas the wild type LPS was ineffective. Likewise, MPL and DPL suppressed the PFC response in C3H/HeJ mice when given prior to antigen.

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Materials and Methods

Mice. Balb/c mice originally purchased from the Charles River Laboratory, Wilmington, MA were bred in our animal facilities by brother and sister matings. The C3H strains were also bred in our facilities, as well as purchased from Jackson Laboratory, Bar Harbor, ME. The young adult mice used were males between 6 and 12 weeks of age. Aging mice of the Balb/c and C3H/HeJ strains were males between 17-26 months old. C57Bl/10 ScN mice were purchased from Harlan Sprague-Dawley Laboratories, Madison, WI.

Antigen. Sheep erythrocytes (SRBC) were purchased from Wilfer Laboratories, Stillwater, MN. They were washed in Hank's balanced salt solution (HBSS) three times before being suspended in buffered saline, pH 7.2. Injection of antigen was performed ip. on day 0.

Endotoxin and lipid A's. Endotoxin (Et) from a mutant strain of S. minnesota, strain M-7 was prepared by Dr. A. Nowotny by the phenol-water extraction procedure described by Westphal and Jann (19). Monophosphoryl lipid A from S. minnesota strain R595 and diphosphoryl lipid A from S. typhimurium strain G30/C21 were obtained from Ribi Immunochem. Research, Inc. (Hamilton, Montana). Isolation and characterization of the MPL and DPL have previously been described elsewhere (13,14). Stock solutions of MPL and DPL were prepared by dissolving 2 mg quantities in 1 ml volumes of pyrogen-free water containing 0.5% triethylamine. The solutions were clarified to slight opalescence after brief warming in a 50-60°C water bath and sonication. These stock solutions were further diluted with saline or with saline containing the antigen to the desired concentration of adjuvant and antigen and injected ip. For adjuvant studies the compounds were injected ip. on day 0 along with antigen and were given ip. 1 or 2 days prior to antigen in order to induce suppression.

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Spleen cell preparation. Spleens were removed from mice sacrificed by cervical dislocation on day 4. Single cell suspensions were prepared and allowed to settle, and the cells were then washed 2X in HBSS before resuspending in saline to a final concentration of 5×10^5 cells/ml for use in the hemolytic plaque assay.

Hemolytic plaque assay. Antibody production was measured by utilizing a modified hemolytic plaque assay (20). Briefly, 60 x 15mm Nunc plastic culture dishes were coated with 2 mls of poly-L-lysine (50 µg/ml). After 15 min. the plates were washed with saline and 2 mls of washed SRBC (diluted 1:20) were added. After 15 minutes the plates were swirled, allowed to settle for another 15 minutes, rinsed with buffered saline and finally 1.5 ml of phosphate buffered saline pH 7.2, were added to each plate along with 2.5 x 10^5 spleen cells and incubated in the presence of complement at 37C for 1 hour, after which plaques were counted.

Preparation of oil-in-water emulsions. When MPL was tested in combination with trehalose dimycolate (TDM), an adjuvant of mycobacterial origin obtained from Ribi ImmunoChem Research, Inc., it was incorporated together with ovalbumin (Sigma Chemical Co., St. Louis, MO) into light mineral oil (Drakeol 6-VR Penreco, Inc., Butler, PA). The adjuvant and antigen were then dispersed as minute oil droplets in saline containing 0.2% Tween 80 detergent. Briefly, MPL and TDM were solublized separately in chloroform-methanol (C:M; 4:1) at 10 mg/ml. For each 1.0 ml of final emulsion to be prepared 25 µl of TDM in chloroform-methanol and 25 µl or 12.5 µl of MPL in chloroform-methanol were dispensed into a 10 ml glass tissue grinder (Potter-Elvehjein type). The chloroform-methanol was evaporated by blowing a gentle stream of nitrogen over the solution leaving the TDM and MPL dried to the inside of the grinder tube. At this point, 250 µg of dry powdered

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ovalbumin and 20 μl of mineral oil were added to each 1.0 ml final volume of emulsion being prepared. Next the teflon pestle rod was clamped in the chuck of a power drill and the pestle was inserted into the grinding tube. The oil and antigen/adjuvant mixture were ground into a paste on the wall of the lower 1/3 of the grinding tube. Grinding was continued for 2-4 min at room temperature using a drill speed of 800-1200 rpm. While leaving the pestle in the grinding tube, the desired final volume of 0.2% Tween-80 in slaine was added. The oil paste-mixture was emulsified into the Tween-80 by slowly moving the pestle up and down in the grinding tube over a period of 4 min with a drill speed of 800-1200 rpm. The emulsion was decanted into a vial and used for injection. Typically mice were injected subcutaneously (flanks) with 0.2 ml of 2% oil-in-water emulsion containing 50 μg ovalbumin, 50 μg TDM, and 50 μg or 25 μg of MPL.

Ovalbumin was incorporated into complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) as follows. Two ml of ovalbumin in saline at a concentration of 500 μg/ml were emulsified with 2 ml of CFA or IFA (Colorado Serum Co.) by repeatedly drawing the mixture into a 10 ml syringe and vigorously ejecting it through an 18-gauge needle. This process was continued until a drop of the emulsion placed on the surface of 4°C water remained intact. Mice were injected subcutaneously with 0.2 ml of emulsion.

ELISA for mouse serum antibodies to ovalbumin. Ovalbumin (Sigma) at 10 μg/ml in 0.05M carbonate-bicarbonate buffer, pH 9.5, was added in 100 μl volumes to the wells of a 96-well Immulon 2 (Dynatech) flat-bottom plate. The plates were incubated at room temperature overnight and the wells washed with distilled water before use to remove unbound ovalbumin. After washing, the plate wasinverted and shaken vigorously to remove excess water. Mouse sera to

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be examined for antibodies to ovalbumin were diluted in 2-fold steps in assay buffer.

The assay buffer consisted of the following: 10mM TRIS (pH 7.5) containing 25% heat inactivated (56°C for 30 min.) fetal calf serum; 2.0 mg/ml EDTA (tetrasodium salt); 0.1% polyoxyethylene sorbitan monolaurate (Tween 20); and 50 µg/ml thimersol (ethylmercurithio-salicylate as a preservative. Each serum dilution was tested by placing 100 µl volumes into each of 2 wells of the ovalbumin coated plate. After addition of all serum dilutions to be tested, the plates were sealed and incubated in a 37°C incubator for 60 min. After incubation, the plates were washed with distilled water as above to remove unbound antibody. After inverting and shaking the plates to remove excess water, 100 µl volumes of horseradish peroxidase conjugated goat anti-mouse IgG (Kirkegaard-Perry) at 1 µg/ml in the assay buffer described above were added to the wells. The plates were incubated at 37°C for 60 min. and washed as before to remove unbound reactants. Finally, 100 µl volumes of chromagen solution containing 0.4 mg of O-phenylenediamine per ml and 0.01% H_2O_2 in 0.1M citrate phosphate buffer (pH 5.0) were added to the wells. After 15 min. incubation at room temperature, the enzymatic reaction was stopped by the addition of 50 µl of 1 N H_2SO_4 to each well. The colored product was measured at 490nm using a Dynatek plate reader. The reader was zeroed by reading wells that had received assay buffer only during the first incubation step.

Statistical Analysis. Data were analyzed by a BMDP statistical program for a one sample "t" test after taking the log_{10} of the ratio of each pair of groups to be compared.

Results

The ability of the mono and diphosphoryl compounds to enhance PFC to SRBC
in young adult, male, Balb mice is shown in Table 1. Both compounds exhibited
strong adjuvant activity, with the MPL under these conditions appearing more
active than the DPL. When using a high dose of antigen (10^8 SRBC), low doses
(1-10 µg) of both DPL and MPL were ineffective while 50 µg of DPL and 25 µg of
MPL increased PFC formation significantly (data not shown).

The ability of MPL to enhance antibody formation against a purified
protein antigen, ovalbumin, is illustrated in Table 2. MPL alone caused a
16-fold increase in the ELISA titer on day 14, as compared to a 4-fold
increase using complete Freund's adjuvant (CFA) or incomplete Freund's
adjuvant (IFA). A striking increase in the response was observed when the
antigen was incorporated together with MPL and TDM into an oil-in-water
emulsion. Titers were increased up to 512-fold by such preparations.

Significant non-specific suppression of PFC responses also was exerted by
each of the two compounds when given 1 day prior to antigen as seen in Table 3.
Thus, both the toxic DPL and the non-toxic MPL proved to be good
immunomodulators, enhancing under different conditions both the helper and
suppressive arms of the immune response.

It was of interest to determine whether the immunomodulating properties
of the DPL and MPL could be expressed in the endotoxin hypo-responsive mouse
strains, C3H/HeJ and C57Bl/10 ScN. Several investigators have noted recently
the capacity of cells from these strains to respond to certain lipid A
moieties of endotoxin despite being unable to respond to the intact
lipopolysaccharide (21,22). The data for the C3H/HeJ strain and its
responsive counterpart, the C3H/HeN strain are shown in Table 4 and reveal
that both compounds exerted an adjuvant action on the helper branch of PFC

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formation in this LPS low-responding strain. As expected, the native endotoxin used as a control did not exert enhancement in the C3H/HeJ strain. Furthermore, both compounds also acted as immunosuppressants in this strain when given 2 days before antigen (Table 5). In addition, both MPL and DPL enhanced antibody responses in C57Bl 10/SCN mice when given with antigen (Table 6), whereas neither preparation suppressed the response in this strain when given a day before antigen (data not shown).

Models of immunodeficiency generally permit the expression of adjuvanticity most effectively. Consequently, the DPL and MPL were tested for adjuvant properties in the aging Balb mouse, a model for immunodeficiency used routinely in our laboratory. The data are recorded in Table 7 and illustrate an enhancing action in mice receiving either compound. Confirmation of the capacity of DPL and MPL to enhance the immune response of aging mice was achieved using the C3H strain, Table 8, where strong enhancement again was clearly evident with both compounds.

Discussion

The isolation of purified mono and diphosphoryl lipid A's offered the opportunity to study the role of toxicity in the profound adjuvant properties previously described for lipopolysaccharides. Detoxification of LPS with retention of its beneficial properties has long been sought. Several studies have reported success in reducing lethality and pyrogenicity (9-12). In early studies Johnson and Nowotny (9) showed that the toxicity of endotoxins could be diminished by treating with either boron trifluoride, potassium methylate or pyridinium formate with retention of partial adjuvant activity. In addition, McIntire et al. established that LPS treated with o-pthalic anhydride reduced toxicity without loss of adjuvant action (10). Chedid et al. (12) have confirmed this observation. Despite these successes a major

problem remains in that certain species including humans, horses, and rabbits are extremely sensitive to trace amounts of residual toxic endotoxins that might still be present in detoxified samples (9).

Recently, a monophosphoryl lipid A fraction of endotoxin (MPL) was isolated and characterized as a non-toxic defined entity (13-16). In contrast to the DPL, the MPL was 2,000-20,000 times less toxic as measured by LD_{50} in rabbits as well as 1000 fold less pyrogenic in rabbits (8). Although the MPL was essentially non-toxic, it retained the ability to act as a strong mitogen and anti-tumor agent (8). Our data extend these beneficial properties and show that the MPL like the native endotoxin also acts as a potent immunomodulating agent stimulating both the enhancing and in certain strains the suppressing arms of the immune system. Its activities in these respects appeared to equal or exceed those exhibited by the toxic DPL compound.

A dramatic enhancement of antibody-production was observed when antigen and MPL were combined together with TDM in an emulsion of light mineral oil. The oil-in-water adjuvant emulsion used in this study advantageously replaces the Freund's complete type water-in-oil type adjuvant (FCA) inasmuch as the concentration of oil is lowered from 50% to 2% which minimizes the formation of sterile abscesses and persistent nodules at the site of injection. The MPL-TDM emulsion, in contrast to FCA, is devoid of tubercular protein which causes undue allergic reactions upon repeated inoculation and is tolerated when administered intravenously to laboratory animals, cattle and horses (23, and Dr. Jack Ward, personal communication). Data in Table 2 demonstrate that the MPL-TDM oil droplet adjuvant system yielded higher titered antisera than any other adjuvant system studied. Titers elicited with the former adjuvant system remained higher over a longer period and led to more rapid anamnestic responses (Cantrell and Ulrich, unpublished findings). The importance of the

oil droplets may be to provide a "depot" for storage and slow release of the adjuvant compounds (24).

Most aging mice exhibit a dramatic decrease in the ability to produce antibody in vivo as well as in vitro (25-28). The use of adjuvants to restore humoral immunity in aging mice has been successful in several instances, eg. polyribonucleotide complexes (25) and 8 mercaptoguanine (26). On the other hand, endotoxic lipopolysaccharides, when tested in this immunodeficient model in the latter study were ineffective in exerting their immunoenhancing capabilities (26,28). Thus, spleen cells from aging mice were unable to respond to antigen alone, whereas addition of 8 mercaptoguanine evoked a marked enhancement of antibody formation to levels seen in young adult mice. Under these conditions, however, wild type LPS was incapable of providing a signal for enhanced antibody formation. Yet, as is shown herein in Tables 7 and 8, both the MPL and DPL fractions were fully capable of restoring antibody production in aging mice. Consequently, certain structural features of the native LPS may be inhibitory to the signalling capacity of lipid A and require enzymatic removal before rendering the LPS capable of stimulating the immune response. If internalization is required for adjuvanticity (29), the fractional forms of LPS such as MPL and DPL may be in a micellar state or molecular size more capable of penetrating the membrane than the larger intact LPS molecules. That lymphocyte membrane composition changes occur with increasing age has been documented by others (30,31). In the young mice the putative inhibitory fractions of the LPS may be readily removed by membrane-bound enzymes not present in aging mice. Since both aging and hyporesponsive mice do not respond to native LPS, and do respond to the MPL and DPL fractions, the question is raised as to whether the defect with respect to LPS adjuvanticity is the same in both these models.

Vukajlovich and Morrison earlier have postulated that the presence of the polysaccharide portion of LPS may mask the adjuvant expression of lipid A per se in unresponsive strains. They reported a lipid A rich polysaccharide and protein-free fraction of LPS, isolated by gel filtration chromatography, was capable of inducing mitogenic proliferation in C3H/HeJ splenic cultures (21). Also, the recent studies of Vogel et al. have shown that a chemically defined lipid A precursor from a mutant strain of S. typhimurium also was able to act as a potent mitogen on C3H/HeJ spleen cells (22). In addition, the precursor stimulated spleen cell cultures from C57B1/10 ScN mice, another endotoxin hyporesponsive strain. However, LPS from an Rc mutant prepared by phenol-water extraction was ineffective in stimulating either of these strains.

Non-specific suppression of the immune response when given prior to antigen is a characteristic of a number of adjuvants, notably polynucleotide complexes (32), muramyl dipeptides (33), as well as native endotoxins (5-7,34). The data reported herein document that this is a property also of both the DPL and MPL compounds, with the latter to date representing the minimal basic structure necessary for this activity.

In addition, the data establish that the smallest endotoxin subunit to date capable of activity as an adjuvant or suppressant to antibody synthesis is the MPL. It follows also that KDO or the phosphate group at the reducing end of the glycosamine disaccharide unit is not required for the adjuvant action. Further studies designed to evaluate the role of the different fatty acids in adjuvant activity are in progress.

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TABLE 1

Enhancement of antibody formation by DPL and MPL

in young adult Balb mice.

Compound[a]	Average PFC/Plate					
	Exp. 1		Exp. 2		Exp. 3	
	Control	Exp.	Control	Exp.	Control	Exp.
DPL	4	24	67	128	26	169
MPL		133		226		442

[a] 50 µg DPL or MPL injected ip with 10^7 SRBC. PFC assayed on day 4. Each data point is the average of three mice assayed individually. $p < 0.03$ for both DPL and MPL as compared to control values.

TABLE 2

ELISA activity of serum from (C57Bl/10 x BALB/c) F_1 mice

mice given 50 μg ovalbumin alone or with adjuvants.

Adjuvant	Dose (μg)	Form	ELISA titers (14 days after immunization)
none	none	aqueous	400
MPL	100	aqueous	6,400
CFA[a]	—	emulsion (50% oil)	1,600
IFA[a]	—	emulsion (50% oil)	1,600
MPL + TDM[b]	50+50	emulsion (2% oil)	102,400
MPL + TDM[b]	25+50	emulsion (2% oil)	204,800
TDM	50	emulsion (2% oil)	12,800

[a]CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant.

[b]TDM, Trehalose dimycolate.

TABLE 3

Suppression of Antibody by MPL and DPL Given Prior to Antigen.

| Compound[a] | Average PFC/plate | | p Value |
	Control	Experimental	
DPL (11) 50 μg	94	36	.001
MPL (13) 25 μg	101	21	.002

[a]injected ip 1 day before injection of 10^8 SRBC ip.
PFC assayed on day 4.
( ) = number of Balb, 6-10 wk old mice tested individually.

TABLE 4

Enhancement of Antibody Formation by MPL and DPL in the

C3H/HeN and the LPS Hyporesponsive C3H/HeJ Mouse Strains.

Mouse Strain	Compound[a]	Averag. PFC/Plate		p Value
		Control	Experimental	
C3H/HeN	DPL (9)	46	135	.007
	MPL (13)	159	345	.05
	ET (4)	198	367	.02
C3H/HeJ	DPL (9)	62	140	.05
	MPL (9)	114	225	.01
	ET (9)	114	131	.08

[a]Injected ip with 10^7 SRBC on day 0. PFC assayed on day 4.
50 µg DPL and 25 µg MPL or ET used.
( ) = number of mice assayed individually.
ET = phenol water extract from S. minnesota R-7 mutant.

TABLE 5

Suppression of Antibody by MPL and DPL in C3H/HeN

and the LPS Hyporesponsive C3H/HeJ Mouse Strains.

Compound Given on Day	Strain	Compound	Average PFC/plate[*]		p Value
			Control	Experimental	
-1	C3H/HeN	DPL (5) MPL (2) ET (2)	218 269 233	134 178 117	.0001 .008 .02
	C3H/HeJ	DPL (6) MPL (11) ET (12)	222 115 106	51 76 61	.004 .10 .05
-2	C3H/HeN	DPL (12) MPL (14)	232	133 103	.04 .01
	C3H/HeJ	DPL (12) MPL (9)	286 260	156 178	.002 .02

[*]SRBC injected ip on day 0. PFC assayed on day 4.
( ) = number of mice assayed individually.
ET = isolated from S. minnesota R-7 mutant.

TABLE 6

Enhancement of Antibody by MPL and DPL in

C57B1 10/ScN Mice.[*]

Compound[b]	PFC/2 x 10^5 cells[a]					
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	$\bar{x}$	p
PBS	199	29	102	52	96	—
DPL	333	169	246	105	213	0.04
MPL	362	112	238	104	204	0.01
ET	235	62	124	56	119	0.2

[a]Total of 10–11 mice tested individually for each compound; PFC assayed on day 4.

[b]25 µg of each compound injected ip with 2 x 10^7 SRBC. Et = phenol water extract of S. minnesota R-7 mutant.

TABLE 7

Enhancement of Antibody Formation by MPL and DPL

in Aging Balb Mice

Compound[a]	Average PFC/plate		p Value
	Control	Experimental	
DPL (50 µg) (7)	62	403	.026
MPL (25 µg) (13)	116	547	.003

[a] injected ip on day 0 with 10^8 SRBC into 17-24 month old mice.

( ) number of mice tested.

TABLE 8

Enhancement of Antibody by MPL and DPL

in Aging C3H Mice

Compound[a] (50 μg)	Average PFC/Plate					
	Exp. 1		Exp. 2		Exp. 3	
	Control	Exp.	Control	Exp.	Control	Exp.
DPL	10	151	2	382	30	369
MPL		108		174		175

[a] injected ip with 10^7 SRBC with 17-24 mo old male mice.   PFC assayed on Day 4.   All p values = < .05.   3 mice used/value.